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(71) Applicant:

Sebald, Walter, Prof. Dr., 97074 Würzburg, DE

(74) Representative:

Grünecker, Kinkeldey, Stockmair & Schwanhäusser, 80538 Munich

(72) Inventor:

Sebald, Walter, Prof. Dr., 97074 Würzburg, DE:
Nickel, Joachim, 97222 Rimpar, DE

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EP 08 91 985 A1
EP 07 23 013 A2
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(54) Mutein of a Chain of a Protein from the Superfamily of the Growth Factor TGF-β

(57) The present invention relates to muteins of a chain of a protein from the superfamily of the growth factor TGF-β having antagonistic and/or partially agonistic activity.

The factors of the TGF-β superfamily each perform their own specific functions in the organism. Overexpression of these proteins may result in serious consequences for the affected patient, for example ectopic bone formation or psoriasis. In order to be able to counter these pathophysiological effects, inhibitors for these factors are to be developed.

The invention provides muteins of a chain of a protein from the superfamily of the growth factor TGF-β, which exhibit antagonistic and/or partially agonistic activity after forming a homodimer, wherein the muteins are altered at one or more positions, which in the unaltered protein are involved in low-affinity binding of the protein to the receptor thereof.

The muteins according to the invention are of interest for the prophylaxis and treatment of diseases induced by an overexpression of factors of the TGF-β superfamily.

Description

[0001] The present invention relates to muteins of a chain of a protein from the superfamily of the growth factor TGF-β having antagonistic and/or partially agonistic activity, to derivatives of a protein from the TGF-β superfamily, to pharmaceutical compositions comprising the muteins and/or derivatives according to the invention, and to nucleic acids coding for the muteins or derivatives thereof.

[0002] The protein family of the TGF(transforming growth factor)-β comprises a large number of structurally related polypeptide growth factors, each of which regulates a fascinating series of cellular processes, including cell proliferation, cell line determination, differentiation, mobility, adhesion and cell death. The factors are expressed in keeping with complex time-based and tissue-specific patterns and play a key role in the development, homeostasis and repair of nearly all tissues in eukaryotic organisms, from the fruit fly to humans. Overall, these factors are in charge of a significant portion of the intracellular signals, which determine the fate of the cell.

[0003] A number of papers in the past few years have contributed to clarifying the TGF-β signal transduction path. Signal transduction involves receptor serine kinases at the cell surface, the substrates of which, the SMAD proteins, migrate into the nucleus after phosphorylation, where they activate the transcription of the target gene in cooperation with DNA-binding partners. The multifunctional nature of TGF-β and the additional factors that are part of the TGF-β superfamily appears to be based on the interaction of different receptors, SMAD proteins, and DNA-binding proteins. Interference with this signal transduction path is the cause of different forms of human carcinomas and developmental disabilities.

[0004] The TGF-β superfamily comprises different subfamilies, each having two to four members. A detailed overview of the different subfamilies and the properties thereof is provided in Massague (1998), for example.

[0005] The following Table I provides an overview of several of the most important members known today of the TGF-β superfamily, of which there are a total about of 20 and which as “bone morphogenetic proteins” and “growth and differentiation factors” control the production and regeneration of tissue in the adult organism and are significantly involved in early and late stages of embryonal development.

Table I

Name	% Identity	Typical Activities	Pathophysiology
BMP -2 BMP -4	100 92	Bone and cartilage development; embryonal development; apoptosis	Ectopic bone formation
BMP-5 BMP-6 BMP-7/OP1 BMP-8/OP2	61 61 60 55	Contribution to the development of nearly all organs	Ectopic bone formation; psoriasis (BMP-6)
GDF-5/CDMP1	57	Cartilage formation in	

GDF-6/CDMP2 GDF-7	54 57	the development of limbs	
GDF-8/myostatin	41	Homeostatis of skeletal muscles	Muscle degeneration?
TGF-β1 TGF-β2 TGF-β3	35 34 36	Growth inhibition, synthesis of extracellular matrix	Fibrosis, scarring

[0006] While the primary amino acid sequences of the members of the TGF-β superfamily in part have relatively little agreement among each other, as is apparent from the Table, all proteins of the different subfamilies have common structural characteristics. For example, all proteins of the dimer superfamily are composed of two usually identical monomers. Another commonality is the signal transduction path: All TGF-β-like protein factors signal through cellular receptors composed of two different types of serine kinase receptor chains. The type I chain is defined by a cytoplasmatic GS box and a serine kinase that activates the cellular SMAD 1 and 5 signaling proteins. The type II chain activates a type I receptor serine kinase by transphosphorylating the GS box segment. The small receptor ectodomains of the type I and/or II chains, each 120 to 150 amino acids long, exhibit only limited similarities among each other and, in addition, different chains of the same type are preserved relatively little. A common characteristic of all known receptor chains of the TGF-β superfamily, however, are four preserved disulfide bridges; additional disulfide bridges and the positions of a few amino acid residues seem to be characteristic of either the type I or type II receptor proteins.

[0007] The members of the TGF-β superfamily, however, fall into two groups with respect to the binding mechanisms thereof, namely that of the TGF-β/activin-like proteins and that of the BMP(bone morphogenic protein)-2 like proteins.

[0008] For the namesake of the superfamily, which is to say the TGF-β, an ordered sequential mechanism of binding to the cellular receptors thereof has been described. First, an external ligand binds to the type II receptor chain, and then within the membrane the recruitment of the type I receptor into the complex takes place. Accordingly, the type II chain represents the high-affinity receptor for TGF-β. The interaction of activins with the receptors thereof seems to employ a comparable mechanism. The members of the TGF-β superfamily, the binding mechanism of which agrees with that described for TGF-β, were therefore given the collective term of TGF-β/activin-like proteins. These include all members of the TGF-β superfamily that have a fourth disulfide bridge in the N-terminal segment. Based on knowledge available today, these are TGF-β1, TGF-β2, TGF-β3, all activins and inhibins and also BMP-11 and GDF-8.

[0009] The binding of BMP-2 to the cellular receptors thereof, however, follows a mechanism that differs from that established for TGF-β/activin-like proteins. In contrast to TGF-β, the high-affinity receptors for BMP-2 are the type I chains BMPR-IA, BMPR-IB, and possibly also ActR-I. Type II chains can likewise bind solute BMP-2, but with considerably lower affinity. From this it has been concluded that the sequential order of type I and type II receptor interaction with BMP-2 is reversed compared with that of the order established for TGF-β. The findings gained for the TGF-β/activin-like proteins can therefore not be applied to BMP-2 and factors having similar mechanisms, such as BMP-4, BMP-5, BMP-6, and BMP-7, GDF-5, GDF-6, GDF-7 (BMP-2-like factors).

[0010] For none of the type I or type II receptors has so far an epitope been localized and characterized on the corresponding ligand. Mutant proteins that exhibit an altered biological activity and receptor binding affinity have been constructed and analyzed for TGF-β1 and activin A. For BMP-2, synthetic peptides have been described, which correspond to the loops of BMP-2 and inhibit BMP-2 activity (EP 691 349).

[0011] All factors of the TGF-β superfamily, including the closely relative representatives of a subset, perform their own specific function in the organism, which manifests itself in the cell- and stage-specific expression of the genes and in the effects of mutations. In mammals, the inactivation of BMP or GDF genes may result in death in different embryonal stages (BMP-2, BMP-4) or in the perinatal period (BMP-7); in addition, specific changes in the development of skeletal elements (GDF-5, BMP-5) have been observed. Overexpression of the proteins may result in ectopic bone formation (BMP-2, BMP-4 and others), psoriasis (BMP-6), neurite production (GFD-5) or the regeneration of ischemic renal damage (BMP-7). An overexpression of GDF-8 (myostation) may result in muscular atrophy. The TGF-β-induced proliferation of the extracellular matrix is also a problem with fibrosis, scarring, or cirrhosis.

[0012] In order to be able to prevent or counteract a pathophysiological effect of the proteins from the TGF-β superfamily, it would thus certainly be desirable to develop inhibitors for these factors.

[0013] It is therefore the object of the invention to provide means by which the pathophysiological effects of members of the TGF-β superfamily can be prevented.

[0014] According to the invention, this object is achieved by a mutein of a chain of a protein from the superfamily of the growth factor TGF-β, wherein the mutein exhibits an antagonistic and/or partially agonistic activity after forming a homodimer, the mutein being altered at one or more positions thereof, which in the unaltered protein are involved in low-affinity binding to the receptor thereof.

[0015] An antagonist shall be interpreted as proteins according to the invention, which bind to the receptors for the natural proteins of the superfamily of the growth factor TGF-β, however by the binding thereof do not elicit the normal biological subsequent reactions. As a result, no signal transduction takes place after the binding of an antagonist. “Partially agonistic activity“ in the context of the present invention shall be understood as an activity, which to a certain extent does elicit the normal biological subsequent reactions, however the scope of these subsequent reactions is considerably less than that of a subsequent reaction elicited by a natural protein. Partially agonistic activity in the context of the present invention shall therefore be understood as an activity that is less than 80%, preferably less than 50%, and particularly preferred less than 25% of the activity of the corresponding natural protein. The activity may be determined, for example, by the C2C12 assay, which is described below in the section “Materials and Methods”.

[0016] “Low-affinity binding“ in the context of the BMP-2-like subfamily shall be understood as binding that results in half-maximal saturation of the receptor protein only for a concentration of the ligand of 10 nM or more, frequently even of more than 100 nM or 1 μM. Contrary thereto, “high-affinity binding“ shall be understood as binding that results in half-maximal saturation of a receptor protein already at a concentration of the ligand of less than 10 nM, frequently already at less than 1 nM. Saturation as a function of the ligand concentration can be measured using a biosensor system, as is described in Example 4.

[0017] In conjunction with the subfamily of the TGF-β/activin-like proteins, “high-affinity” binding enables the binding of the ligand to receptor type II even in the absence of receptor type I, which can be detected in complete cells by chemical cross-linking with radioactively

labeled ligands. Contrary thereto, “low-affinity“ binding of the ligand to receptor chain I in the absence of the type II chain is possible with extremely low efficiency and is amplified in the presence of the type II chain. This can likewise be checked by crosslinking with radioactively labeled ligands (for example, refer to Massague, 1998; Wuytens et al., 1999).

[0018] An “unaltered protein“ shall be understood as a growth factor from the TGF-β superfamily in the form in which it is found naturally in a mammal and performs the biological activity. The statement “at one or more positions“ shall mean that in the mutein optionally only a single amino acid is modified, however that also a larger number of amino acids may be altered, for example where farther reaching deletions were carried out. In preferred embodiments, between 1 and 50, particularly preferred 1 to 25 or 1 to 10, most preferred between 1 and 5 amino acids are altered.

[0019] According to the invention, the muteins may comprise a deletion of one or more amino acids, wherein the deletion of several amino acids may relate to several positions of the protein chain. However, muteins in which one or more amino acids are substituted by other amino acids are preferred, wherein the several amino acids may be adjacent or not adjacent. Non-conservative substitution is preferred, with the substitution by an amino acid having a different charge or different size being particularly preferred. Here, the following explanation shall be provided: In principle, differentiation is made between four physicochemical groups into which the naturally occurring amino acids are divided. The group of basic amino acids includes arginine, lysine, and histidine. The group of acid amino acids includes glutamic acid and asparginic acid. The uncharged/polar amino acids comprise glutamine, asparagine, serine, threonine, and tyrosine. The non-polar amino acids include methionine, phenylalanine, tryptophane, cysteine, glycine, alanine, valine, proline, leucine, and isoleucine. Non-conservative substitution in this context shall mean the replacement of an amino acid with an amino acid in a different physicochemical group. The replacement of an amino acid of a first group with an amino acid of a second group is particularly preferred, the amino acids of the second group having a different charge than the amino acids of the first group.

[0020] Also preferred is the replacement of a large amino acid with one of the smaller amino acids glycine, alanine or serine. Furthermore, the replacement of one of the smaller amino acids with one of the larger amino acids tryptophane, tyrosine, phenylalanine, leucine, isoleucine or glutamine is preferred.

[0021] In a third embodiment, one or more amino acids are inserted. Insertions of several amino acids can take place at one position or several positions of the chain.

[0022] In a further embodiment, one or more of the listed amino acid residues are chemically modified. A modification can be, for example, the covalent bond with one or more residues, which are selected from the following groups: Carboxylic acids, amines, polyethylene glycol, biotin, and sugar (DeSantis et al., 1999).

[0023] In a preferred embodiment, the mutein is derived from a chain of a BMP-2-like protein. The family of the BMP-2-like proteins includes the BMP-2 subfamily, BMF-5 subfamily, and GDF-5 subfamily (refer to the classification of these families by Massague (1998)). As is also apparent from Table I, the members of the BMP-2 subfamily exhibit a 92% identity among each other, while the members of the BMP-5 and GDF-5 subfamilies exhibit an identity of 54 to 61%, relative to BMP-2. With respect to the members of these three subfamilies, it is assumed that they follow the above-mentioned reaction mechanism that has been found for BMP-2, which is to say that contrary to the TGF-βs or activins they first bind with high affinity to the type I chains BMPR-IA, BMPR-IB and possibly also ActR-I.

[0024] Surprisingly it was found that muteins having partially agonistic or antagonistic activity are those muteins for which in the protein chain derived from BMP-2, BMP-4, BMP-5 and the like at least one amino acid was deleted, substituted or modified in the binding epitope for the natural BMP receptor II, or at least one amino acid was inserted into the binding epitope. As part of the present invention, the inventors determined the binding epitopes for all receptors involved. It was found that the amino acid positions of BMP-2 determining the binding affinity for either the BMPR-IA or BMPR-II receptor chains represent two non-overlapping sets. As FIG. 1 shows, these determinants are distributed along the entire BMP-2 sequence. The space-filling model of FIG. 5 indicates that the functional residues form two separate epitopes on the surface of the homodimeric BMP-2 molecule.

[0025] The determinants for BMPR-IA interactions are located in the first epitope comprising residues from the two subsets. FIG. 5 shows the amino acid residues of the first epitope on a first subset in italics, while the amino acid residues of the first epitope on a second subset are identified with regular letters. The epitope is highly discontinuous and comprises residues in the β1-sheet, the loop before helix α3 and helix α3 of a monomer, and also parts of the long ω-loop between sheets β-2 and β-3 and the sheet β-8 of the other monomer. One of the monomers thus provides residues V26, D30, and W31 in the β-sheet regions β-2 and β-3 and also the residues K101 and Y103 in the β-sheet region β8. The other monomer contributes residues I62, L66, N68, and S69 in helix α3 and residues F49, P50, A52, and H54 in the region in front of helix α3. Due to the spatial structure of the monomer, this epitope is referred to as the "wrist" epitope. The monomers are compared to an open hand, wherein the central helix α3 represents the wrist and two β-sheets arranged side by side represent the four fingers, with loops 1 and 2 corresponding to the finger tips of each pair of fingers. The N-terminal segment is located at the position of the thumb. Consequently, the first epitope disposed around the central α-helix is the wrist. The dimensions thereof are approximately 2 x 2.5 to 3 nm. This area is compatible with the function as a high-affinity interaction site.

[0026] The second epitope disposed at the back of the hand near the outer finger segments is in charge of the low-affinity binding of BMP-2 to the BMPR-II. It only comprises amino acid residues of one subset and is also referred to as the "knuckle" epitope. The amino acid residues A34 and H39 occur in β-3 and β-4, the amino acid residues S88 and L90 occur in β-7 and L100 in β-8. With respect to the amino acid residue E109, the inventors assume that it is a further important contact amino acid residue. The second epitope seems to be much smaller than the first epitope, since many amino acid residues at the boundaries of the second epitope can be modified without detectable effects on receptor binding or biologically activity. However, it cannot be excluded at the present time that the epitope comprises further functional amino acid residues.

[0027] The two epitopes are separated from each other functionally and spatially. All binding determinants were found to be specific either for BMPR-I (type I) or BMPR-II/ActR-II (type II). In the case of BMP-2, antagonistic muteins were found only for the knuckle epitope. The different epitopes are defined by binding determinants and delimited from each other by neutral residues. They represent non-overlapping regions on the surface of the established three-dimensional structure of BMP-2. However, it cannot be excluded that cooperative effects take place during type I and type II receptor binding. The wrist epitope and the knuckle epitope are separated from each other only by the thickness of a β-sheet, which may change conformation after bonding to the ectodomain and in this way may act as an agent for cooperative effects. The spatial separation of the epitopes further suggests that each

symmetry-related part of the dimeric BMP-2 molecule comprises a pair of functional epitopes and that two independent wrist epitopes and two independent knuckle epitopes can bind a total of four receptor chains. A complex between a BMP-2 and two BMPR-IA ectodomains has already been identified (Kirsch et al., 2000 (c)).

[0028] None of the epitopes, however, exhibits the typical charge characteristics recently discussed for receptors (Griffith et al., 1996).

[0029] Without being bound to this theory, it is assumed that the BMP-2 antagonists according to the invention most likely are the result of an ordered sequential binding mechanism that causes the receptor activation. According to the model, the antagonist blocks the high-affinity type I receptor chain with the intact wrist epitope thereof, and the knuckle epitope altered by substitution, deletion, modification or insertion prevents the subsequent oligomerization with low-affinity type II receptor chains. The comparatively low IC_{50} of the antagonists and the efficient competition thereof with BMP-2 for receptor binding indicate that it is predominantly type I chains that control the binding of BMP-2 to the entire receptor complex, possibly in that they determine the association rate for BMP-2. The half-life value of the complex between BMP-2 and the type 1 receptor of more than 30 minutes most likely causes the binding to the cellular receptor to be irreversible. An interesting observation in this context is the low residual activity of the highly antagonistic muteins A34D and L90A in the C2C12 assay, when taking into consideration that binding to the ectodomains of the type II receptor chains is reduced only approximately 5- to 15-fold. It is possible that the simultaneous binding of two type II chains is necessary for efficient receptor activation and therefore a decrease in binding affinity has a greater effect.

[0030] Since the other BMP-2-like proteins activate the corresponding receptors thereof based on the same mechanism as was shown for BMP-2, which is to say by way of a high-affinity wrist epitope and a low-affinity knuckle epitope, antagonistic muteins of these proteins can also be produced by amino acid substitutions in the knuckle epitope.

[0031] In preferred embodiments, one or more amino acid residues forming the surface-exposed regions in the β-sheet structures β-3, β-4, β-7, β-8, or β-9 are modified. These surface-exposed residues are the following:

β-3: V33, A34
between β-3 and β-4: P35, P36;
β-4: G37, Y38, H39;
after β-4: F41, Y42;
β-6: T82, E83, L84, S85;
β-7: A86, I87, S88, L90;
β-8: K97, V98, V99, L100;
β-9: V107, E109, G110.

[0032] In a first embodiment, one or more of the listed amino acid residues are deleted individually or in groups of up to 5 amino acids. Preferably amino acids are deleted for which interaction with the BMP receptor II is proven or the deletion of which affects the conformation of the knuckle epitope. By combining a substitution with an insertion and/or deletion, or also by combining an insertion with a deletion, further muteins can be produced, which optionally have lower affinity for the BMP receptor II.

[0033] A further possibility for arriving at antagonistic or partially agonistic muteins starting from the known sequence of a monomer for BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, GDF-5, GDF-6, or GDF-7 is to insert one or more amino acids into the surface-exposed regions of

the knuckle epitope. In principle, these amino acids also must fulfill the purpose of weakening or preventing binding to the BMP receptor II.

[0034] In a preferred embodiment, the mutein is a chain of a BMP-2-like protein, wherein one or more of the following amino acids from BMP-2, or amino acids corresponding thereto from a different BMP-2-like protein, are substituted by other amino acids: V33, A34, P35, P36, G37, Y38, H39, F41, Y42, T82, E83, L84, S85, A86, I87, S88, L90, K97, V98, V99, L100, V107, E109, and G110.

[0035] The following Table II provides an overview of preferred replacement amino acids for the above-mentioned amino acid residues:

Table II

Amino acid residues (BMP-2)	Replacement amino acids	Preferred replacement amino acids

(see original for remaining table information)

[0036] From literature it is known that different members of the BMP subfamilies 2 and 5 and of the GDF subfamily 5 have identical arrangements of the cysteines crucial for the tertiary structure, albeit overall there is relatively little homology between these proteins. Accordingly, while taking these preserved positions into consideration, the amino acid positions that correspond to a defined amino acid in BMP-2 can be determined for the remaining members of the subfamilies listed. For example, the BMP-2 position V33 in BMP-7 corresponds to isoleucine, A34 is alanine, P35 is proline, P36 is glutamate, H39 is alanine, S88 is serine, L90 is leucine, V98 is valine, L100 is leucine, and E109 is arginine. FIG. 6 shows an alignment of the sequences of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, GDF-5, GDF-6, GDF-7, GDF-3, GDF-1, BMP-10, GDF-2, BMP-15, GDF-9B, GDF-9, BMP-3, GDF-10, Act-A, Act-B, Act-C, BMP-11, GDF-8, TGF-β1, TGF-β2, TGF-3, Inh-a, MIS, and GDNF carried out with the "MultAlin" program and indicates the amino acids that correspond to a defined BMP-2 amino acid in other members of the TGF-β superfamily. The positions determined by the comparison to BMP-2 can likewise be modified by substitution, deletion, or chemical modification. Likewise, the epitopes may lose binding affinity by insertion, wherein insertions immediately before or after the positions listed are preferred.

[0037] The alteration of the positions mentioned for BMP-2 in members of the subfamilies BMP-5 and GDF-5 by way of non-conservative substitution, deletion, or chemical modification in each case results in muteins having altered, which is to say typically reduced, binding properties for BMPR-II or for another type II receptor.

[0038] The invention further relates to muteins having at least 50% identity at the amino acid level with a growth factor from the TGF-β superfamily and additionally exhibiting antagonistic and/or partially agonistic activity. As a result, muteins, the amino acid sequence of which differs from the amino acid sequence of the corresponding natural protein chains even in regions that are not critical for antagonistic and/or partially agonistic activity, are also included.

[0039] The term "identity" known to the person skilled in the art denotes the degree of relationship between two or more DNA molecules or two or more polypeptide molecules,

which is determined based on the agreement between the sequences. The "identity" percentage is obtained from the percentage of identical regions in two or more sequences, while taking gaps or other special sequence characteristics into consideration.

[0040] The identity of related polypeptides or DNA molecules can be determined by using known methods. In general, special computer programs that include algorithms taking the special requirements into account are employed. Preferred method for determining identity first produce the largest agreement between the sequences analyzed. Computer programs for determining the identity between two sequences include, but are not limited to the GCG program package, to include GAP (Devereux, J., et al., Nucleic Acids Research 12 (12): 387 (1984); Genetics Computer Group University of Wisconsin, Madison, (WI)); BLASTP, BLASTN and FASTA (Altschul, S. et al., J. Molec Biol 215: 403/410 (1990)). The BLAST X program may be obtained from the National Centre for Biotechnology Information (NCBI) and other sources (BLAST Handbuch [Handbook], Altschul S., et al., NCB NLM NIH Bethesda MD 20894; Altschul, S., et al., J. Mol. 215: 403/410 (1990)). The known Smith-Waterman algorithm may also be used for determining identity.

[0041] Preferred parameters for the sequence comparison comprise the following:

Algorithm: Needleman-Wunsch, J. Mol. Biol 48: 443-453 (1970)

Comparative matrix: BLOSUM62 from Henikoff & Henikoff, PNAS USA 89 (1992), 10915-10919

Gap penalty: 12

Gap length penalty: 2

[0042] The GAP program is also suited for use with the above parameters. The above parameters are default parameters for amino acid sequence comparisons, wherein gaps at the ends do not reduce the homology value. In the case of very small sequences compared to the reference sequence, it may further be necessary to increase the expected value to 100,000 and optionally reduce the word size to 2.

[0043] Additional examples of algorithms, gap opening penalties, gap extension penalties, comparison matrixes, including those mentioned in the program manual, Wisconsin Package Version 9, September 1997, may be used. The selection will depend on the comparison to be performed and also on whether the comparison is carried out between sequence pairs, in which case GAP or Best Fit is preferred, or whether a comparison is carried out between a sequence and a comprehensive sequence database, in which case FASTA or BLAST is preferred.

[0044] An agreement of 50% determined based on the above algorithms is referred to as a 50% identity. The same applies accordingly for higher degrees of identity.

[0045] In preferred embodiments, the muteins according to the invention have an identity of 60% or more, for example more than 70% or 80%, with the sequence of a chain of mature human BMP-2-like protein. The sequence for mature human BMP-2 can be found in Celeste et al. (1990), for example. Also preferred are muteins having an identity of more than 90, 95, or 97%.

[0046] As mentioned above, in the case of BMP-2, the knuckle epitope is the low-affinity binding epitope, while the wrist epitope is the high-affinity binding epitope for the receptor. The muteins according to the invention may furthermore be derived from a protein of the TGF-β/activin family. However, in this case it was surprisingly found that it was not alterations in the knuckle epitope, but alterations in the wrist epitope that resulted in muteins having antagonistic and/or partially agonistic activity. Accordingly, one or more amino acids in the

wrist epitope are altered in muteins according to the invention that are derived from a protein of the TGF-β/activin family.

[0047] The TGF-β/activity family comprises TGF-β1, TGF-β2, TGF-β3, all activins, inhibins, BMP-11, and GDF-8. Activins known so far include, for example activin βA, activin βB, activin βC, and activin βE. Based on current knowledge, the inhibins include inhibin βA, βB, and βC.

[0048] Also in the case of the muteins derived from the TGF-β/activin family, antagonists or partial agonists can be obtained primarily by altering amino acids in the surface-exposed regions, which is to say those regions involved in binding to the receptor. This involves substantially the helix in front of the β-sheet structure β1, the β-sheet structure β1, the long loop between the β-sheet structures β2 and β3, the loop in front of helix α3, helix α3, and the sheet structure β8. As described above for the BMP-2-like proteins, such alterations can be produced by deletions, substitutions, or modifications, and also by inserting one or more amino acids. The possibilities provided above in connection with the BMP-2-similar proteins can be implemented here accordingly.

[0049] In preferred embodiments, at least one of the following amino acids is altered, which is to say deleted, substituted and/or modified, and/or one or more amino acids are inserted, wherein the position information relates to BMP-2:

K5, S13, V26, G27, W28, N29, D30, W31, P48, F49, P50, A52, D53, H54, N59, I62, V63, L66, N68, S69, V70, K101, Y103.

[0050] As is apparent from FIG. 6, this position information corresponds to the following in the TGF-β:

Y6, N14, L28, G29, W30, K31, missing, W32, P49, Y50, I51, S53, missing, missing, Q57, K60, V61, L64, N66, Q67, H68, E99, L101.

[0051] The three-dimensional structure of the complex between BMP-2 and the type I receptor BMPR-IA (Kirsch et al.; 2000 (c)) shows that these residues are significantly involved in receptor contact.

[0052] The muteins according to the invention can additionally be altered in the regions not essential for binding to the receptor, even if they were derived from the TGF-β/activin-like growth factors. Muteins having an identity of at least 50% with a chain of TGF-β/activin-like growth factor exhibiting antagonistic and/or partially agonistic activity are likewise included.Such muteins can originate, for example, from other mammals, such as mice, rats, rabbits, guinea pigs, bovines, pigs, or sheep. Insofar as such muteins exhibit antagonistic and/or partial agonistic activity in the C2C12 cell assay, they are likewise included in the invention. Muteins having an identity of 60% or more, for example more than 70% or 80% identity with a chain of a mutein from the TGF-β/activin family, are preferred. Even more preferred are muteins having an identity of more than 90, 95, or 97%.

[0053] In a further embodiment, it is provided to produce muteins according to the invention by major alterations of the underlying molecules, for example in that an insertion is performed in addition to a substitution. Conceivable muteins of both subfamilies of the TGF-β superfamily also include an insertion in addition to a deletion, or a deletion in addition to a substitution, or at least one substitution, deletion or insertion in conjunction with a chemical modification. Of course it is also possible for teo alterations of one type to take place, for example a substitution at two different sites, alone or in combination with an alteration of a second type, such as an insertion at another site. Likewise, more than two types of alterations may be combined, for example, in addition to a substitution, both a deletion at one site and an insertion at another site may be present.

[0054] The person skilled in the art knows how to produce the muteins. In addition to conventional protein synthesis, such as the Merrifield synthesis, above all recombinant methods lend themselves for substitution, deletion and insertion. On the basis of the known genes, mutations can be introduced in a targeted manner, for example by oligonucleotide-directed location-specific mutagenesis. Fragments can be deleted or inserted. Alternatively, DNA sequences coding for the muteins may be synthesized de novo.
[0055] Chemical modifications are likewise performed in the manner known to the person skilled in the art. The implementation of chemical modifications on protein chains is described, for example, in DeSantis & Jones (1999).
[0056] In further preferred embodiments, the above-described mutein is covalently bonded to a target-specific molecule. This can be, for example, a heparin-binding epitope, causing greater binding to the glycosaminoglycans of the extracellular matrix or the cell surface (for example, refer to PCT/EP00/00637). Provided that this target-specific molecule is an antibody, for example signal transduction in cells comprising a surface protein that is detected by the antibody may specifically be suppressed. However, the mutein can be provided with target specificity not only by covalent binding to an antibody, but optionally also by covalent binding to a ligand which is specific for a receptor that is only present in the target cell.
[0057] In preferred embodiments, muteins having a target-specific molecule covalently bound thereto are produced by the recombinant expression of a fusion protein, which optionally may comprise a spacer between the mutein-coding sequence and target-specific molecule.
[0058] The invention further relates to derivatives of proteins from the TGF-β family, which as an essential component comprise a mutein according to the present invention and, for forming a dimer, a further chain of a protein from the group of the TGF-β superfamily or a further mutein according to the invention. The derivatives can therefore form both homodimers and heterodimers from muteins according to the invention. In addition, a derivative may comprise a mutein and a natural chain of a protein from the TGF-β superfamily.
[0059] The invention further relates to pharmaceutical compositions comprising at least one protein according to the invention and/or a derivative according to the invention. The invention also includes pharmaceutically acceptable salts thereof. Depending on the nature of the mutein contained therein and depending on the pathological condition to be treated, the pharmaceutical compositions may be provided in the form of ointments, creams, or lotions for topical application, or in the form of solutions or lyophilisates for intramuscular or subcutaneous injections. The formulation and production of the pharmaceutical compositions are carried out in accordance with the prior art and include stabilization, among other things.
[0060] According to a further embodiment, the use of a mutein according to the invention and/or a derivative according to the invention for producing pharmaceutical compositions is claimed. These compositions can be used for the prophylaxis and/or treatment of diseases induced by a protein from the superfamily of the TGF-β growth factor. Examples of such diseases include ectopic bone formation, psoriasis, muscular atrophy, scarring, fibroses, and cirrhoses. In the case of ectopic bone formation, preferably a mutein of one of the growth factors BMF-2 or BMP-4 is used, while in the case of cirrhosis of the liver, for example, preferably a mutein of one or more of the growth factors TGF-β1, TGF-β2 or TGF-β3 or a derivative comprising the same is used.
[0061] In further embodiments of the present invention, antibodies against a mutein according to the invention or a derivative according to the invention are provided. Since the muteins are

characterized by an alteration at surface-exposed regions of the molecule, this also has an effect on the antibody populations reacting specifically with the molecule. Antibodies can be produced in the conventional manner, either by immunizing animals (such as rabbits, mice, or rats) in order to produce polyclonal antibodies, or by the immunization and subsequent immortalization of antibody-producing cells in the case of monoclonal antibodies. The person skilled in the art is very familiar with the methods required therefor, however due to the high phylogenetic invariance of the superfamily in the production of antibodies one must be careful to select a host having growth factors that substantially differ from those against which the antibodies are to be produced.

[0062] The invention further relates to the nucleic acids coding for the muteins according to the invention. They contain a nucleic acid sequence coding for a desired mutein. The nucleic acid sequence for BMP-2 is known, for example, from Wozney et al. (1988) and that for TGF-β2 is known from Madisen et al. (1988). The nucleic acid sequence coding for a mutein primarily differs by the triplets coding for the altered amino acids, which is to say by the absence, replacement or insertion of one or more codons. To the extent that the mutein is a mutein having 50% identity or more at the amino acid level, the corresponding ones for a naturally mature protein chain have a reduced identity. Nucleic acids deviating from this due to degeneration of the genetic code are likewise included. Furthermore, sequences complementary to the nucleic acid sequences coding for the muteins and nucleic acids hybridizing with these complementary sequences under stringent conditions and coding for a mutein which exhibits antagonistic or partially agonistic BMP-2 activity, after forming a homodimer, are included. Stringent conditions in this case shall be, for example, hybridization at 68ºC in 0.5 x SSC. These and additional stringent hybridization conditions can be reviewed in the manual by Maniatis et al., 1989.

[0063] The nucleic acid according to the invention may be genomic DNA, cDNA, synthetic DNA or RNA. Genomic DNAs or cDNAs can be isolated from the corresponding gDNA or cDNA banks using methods known in the prior art. For the isolation of nucleic acids from cDNA banks, tissue- or cell line-specific banks are preferred, for example from U-2 osteosarcoma (OS) banks or prostate adenocarcinoma banks. Synthetic DNA can be produced using known methods, RNA can be isolated either by way of RNA vectors or from mRNA. For the recombinant production of muteins according to the invention, genomic DNA or cDNA will be preferred depending on the expression system, however expression by way of RNA vectors is also not excluded.

[0064] In the case of alterations by substitution, methods known from the prior art may be used to replace the codon coding for the original amino acid. In the case of deletions, codons coding for one or more amino acids are removed, while in the case of insertions codon triplets coding for the desired amino acids are inserted. When selecting codons in the case of a substitution or insertion, the person skilled in the art will strive to take into account the codon use by the host organism provided. The corresponding information is available in the prior art.

[0065] According to the invention, nucleic acids comprising a promoter suited for expression control are also provided, wherein the nucleic acid sequence coding for a mutein according to the invention is controlled by this promoter. The selection of a suitable promoter is again dependent on the expression system selected. The person skilled in the art has the choice between a number of known inducible or constitutive promoters for a wide variety of host organisms.

[0066] For the recombinant expression of the nucleic acid according to the invention, the acid is preferably inserted in a vector. The invention further relates to a vector comprising a nucleic acid according to the invention and host organisms comprising a nucleic acid sequence that codes for a mutein, either directly integrated in the genome or in the form of an autonomously replicating vector. From the prior art, numerous prokaryotic and eukaryotic expression systems are known, wherein the host cells are, for example, selected from prokaryotic cells, for example bacteria, such as E. coli or B. subtilis, from eukaryotic cells, for example yeast cells, plant cells, insect cells, and mammal cells, such as CHO cells, COS cells, or HeLa cells, and from derivatives thereof. From the prior art, for example, certain CHO production lines are known, the glycosylation patterns of which are altered compared to CHO cells. The polypeptides obtained by the use of glycosylation-efficient or glycosylation-reduced host cells have an altered spatial structure, which may be accompanied by an altered biological activity.
[0067] The invention further relates to a method for producing a mutein according to the invention, wherein the method comprises the cultivation of a host cell under conditions suitable for expression and optionally the purification of the expressed mutein using methods known from the prior art.
[0068] The following examples and figures explain the invention, without limiting it thereto.

Descriptions of Figures

FIG. 1

[0069] FIG. 1 shows the sequences for BMP-2, BMP-7, TGF-β2, and TGF-β3, wherein corresponding amino acids are arranged beneath each other. Above the BMP-2 sequence, the amino acid residues altered by substitution are provided. BMP-2 muteins having reduced binding affinity for the type II receptor BMPR-II are indicated by a double vertical line. Altered binding affinities for the type I receptor BMPRA-IA that are based on a reduced association or an increased dissociation rate constant are labeled with a plus (+) or cross (x) at the relevant positions. Single vertical lines indicate that no measurable alterations in the function of the corresponding muteins were found.
The numbering refers to the BMP-2 sequence.

FIG. 2

[0070] FIG. 2 provides information about the biological activity and inhibitory properties of BMP-2 muteins.

(A) After the incubation of BMP-2 or a BMP-2 mutein (250 nM), alkaline phosphatase activity was measured. The response caused by each mutein was expressed in terms of % of the BMP-2 response. The values constitute the mean values (+/- standard deviation) of four measurements.

Muteins having solid symbols have been altered with respect to the BMPR-II interaction thereof, as shown in FIG. 4. Plus or cross symbols indicate muteins having an altered association or dissociation constant for binding to the BMPR-IA receptor chain.
(B) The dosage-dependent induction of the activity of alkaline phosphatase in starved C2C12 cells is indicated for BMP-2 (O) and for BMP-2 muteins (◊), D30K (□) and P50A (Δ). The background absorption of 0.080 +/- 0.020 at 405 nm was not deducted for representing the signal/background ratio.
(C) The inhibition of the induction of alkaline phosphatase activity in starved C2C12 cells was determined after incubation with 250 nM BMP-2 mutein in the presence of 10 nM (O) or 20 nM (Δ) BMP-2. The response obtained in the sole presence of BMP-2 is indicated by a dotted line and a value of 100% was used. The values constitute a mean value (+/- standard deviation) of four measurements.
Muteins having solid symbols have been altered with respect to the BMPR-II interaction thereof. Plus or cross symbols indicate muteins having altered association or dissociation constants for binding to the BMPR-IA receptor.
(D) The inhibition of BMP-2 activity (10 nM BMP-2) by increasing doses of potentially antagonistic (partially agonistic) BMP-2 muteins in starved C2C12 cells. The dosage effect lines of the muteins A34D (O), H39D (□), S88A (Δ), L90A (∇), and L100A (◊) in the presence of 10 nM BMP-2 were obtained after incubation of the cells (3 days) and analysis of the induced alkaline phosphatase activity.

FIG. 3

[0071] FIG. 3 shows the biosensor analysis of the binding of BMP-2 and BMP-2 muteins to (A) type I or (B) type II BMP receptor chains.

FIG. 4

[0072] FIG. 4 shows the interaction of BMP-2 muteins with type I (BMPR-IA) or type II (BMPR-II, ActR-II) receptor ectodomains.
[0073] The rate constants for the association (k_{on}) and dissociation (k_{off}) of a BMP-2 mutein at concentrations of 15, 03, and 45 nM with immobilized BMPR-IA receptor ectodomain were derived from the sensograms shown in FIG. 3(A). The sensograms shown in FIG. 3(B) were evaluated in order to derive the equilibrium binding of 45 nM mutein (EQ_{45}) to immobilized BMPR-II or ActR-II receptor ectodomains. All values were normalized by taking the k_{on}, k_{off} and EQ_{45} values of BMP-2 as the standard.

(A) Equilibrium binding of increasing concentrations of BMP-2 to the type I receptors BMPR-IA and BMPR-IB and to the type II receptors BMPR-II and ActR-II. For the determination of equilibrium binding to the immobilized receptor domains, the sensograms shown in FIG. 3 were evaluated.

(B) Differential binding affinity of BMP-2 muteins to BMPR-II or ActR-II receptors. Equilibrium binding during the biosensor analysis of 45 nM mutein (EQ_{45}) on BMPR-II is plotted against binding to ActR-II. The values are normalized by equilibrium binding of BMP-2 to the corresponding receptors.
(C) Graphical representation of the rate constants for the association (k_{on}) and dissociation (k_{off}) of a BMP-2 mutein with the BMPR-IA receptor. Muteins that have been specifically altered with respect to k_{on} are identified with plus symbols, those specifically altered with respect to k_{off} are identified with cross symbols.
(D) Graphical representation of the association constants (k_{on}) for BMPR-IA binding and equilibrium binding (EQ_{45}) to BMPR-II for the same muteins as in (C). Since both the association constants and equilibrium binding depend on the concentration of the BMP-2 mutein, specific (and concentration-independent) alterations become visible. Muteins having a specific decrease in binding equilibrium are marked with solid circles.

FIG. 5

[0074] FIG. 5 is a space-filling model of BMP-2 (Scheufler et al., 1999), in which the residues of the wrist epitope determining type I receptor binding and the residues of the knuckle epitope determining type II receptor binding are identified. The association is apparent from FIG. 1 and also from the tables and lists on pages 12/13 and 17. Residues of one subunit are labeled in bold and italics, residues of the other with a simple upper-case letter.
[0075] On the smaller inserted schematic drawing, the dimeric protein has been rotated in the plane of the paper by 90 degrees about the longitudinal axis.

FIG. 6

[0076] Sequence association of factors of the TGF-β superfamily. The numbering follows the amino acid sequences of mature human BMP-2.

EXAMPLES

Materials and Methods

Production of recombinant Receptor Ectodomains

[0077] An extracellular domain of human BMPR-IA, comprising the residues 24-142 (ten Dijke et al., 1993), including an N-terminal extension (GSGAMA), was expressed as a soluble thioredoxin fusion protein in E. coli. After thrombin cleavage, the protein was purified using affinity chromatography over BMP-2 sepharose, as described by Kirsch et al., (2000 (a)).

[0078] The extracellular domains of ActR-II (amino acid residues 19-126) (Matzuk and Bradley, 1992), BMPR-II (amino acid residues 27-151) (Rosenzweig et al., 1995), and BMPR-IB (amino acid residues 14-126) (Astrom et al., 1999) were expressed with a C-terminal thrombin cleavage site (LVPRGS) puls a 6x His-tag in Sf-9 insect cells (Pharmingen) in accordance with the manufacturer's instructions. The corresponding DNA sequences were inserted in the BamHI cleavage site of the Baculovirus transfer vector pAcGP67B (Pharmingen). The culture medium, which had been incubated for four days after infection of the Sf-9 cells with a MOI (multiplicity of infection) of 3, was applied to Ni-NTA Agarose (Qiagen) in a washing buffer (50 mM NaH_2PO_4, pH 8.3, 300 mM NaCl, 10 mM imidazole) at 4ºC. The recombinant proteins were eluted with elution buffer (50 mM NaH_2PO_4, pH 8.3, 300 mM NaCl, 300 mM imidazole) and thoroughly dialyzed against high salt HBS buffer (10 mM HEPES, pH 7.4, 500 mM NaCl, 3.4 mM EDTA). Finally, the ectodomains were adsorbed on a BMP-2 sepharose affinity matrix (Kirsch et al., 2000 (a)), washed, and eluted with 4 M $MgCl_2$. The purified proteins were transferred to low salt HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3, 4 mM EDTA), concentrated with YM 10 ultrafiltration members, and stored at -80ºC.

[0079] The purified receptor proteins were N-biotinylated by incubation with equimolar concentrations of Sulfo-NHS-LC-Biotin (Pierce), as described (Shen et al., 1996).

Production of BMP-2 muteins

[0080] BMP-2 cDNA coding for residues 283-396 of the mature BMP-2 protein plus the two N-terminal amino acids MA (Ruppert et al., 1996) was subjected to in vitro cassette mutagenesis (Wang et al., 1997), for which synthetic double-stranded oligonucleotides were employed. The BMP-2 muteins were expressed in E. coli, isolated as inclusion bodies, renatured, and purified as described in Ruppert et al., see above.

C2C12 [alkaline phosphatase (ALP)[Assay

[0081] The promyoblast cells C2C12 (ATCC CRL-1772, Blau et al., 1983) were stimulated at a density of 3 x 10^4 cells per well in a 96-well microtiter plate for 3 days with 1 to 250 nM of each BMP-2 variant in 100 µl DMEM medium containing 2% calf serum and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) at 37ºC in a humidified atmosphere at 5% CO_2. The cells were washed with PBS and then lysed for 1 hour with 100 µl NP40 in ALP buffer (0.1 M glycine, pH 9.6, 1 mM $MgCl_2$, 1 mM $ZnCl_2$). ALP activity was determined by incubating lysed cells for 15 minutes with 100 µl ALP buffer plus 1 mg/ml p-nitrophenyl phosphate and measuring the extinction at 405 nm. An A_{405} extinction unit corresponds to 1.5 nmol p-nitrophenolate production per minute per 3 x 10^4 cells. The results were expressed in terms of mean values that had been obtained in four independent experiments using a standard deviation (SD) of +/- 39%. Inhibition experiments conducted in the presence of 10 or 20 nM BMP-2 exhibited higher standard deviations, which are shown in the corresponding figures.

Biosensor Interaction Analysis

[0082] The BIA2000 system (Biacore) was used to record the binding of BMP-2 muteins to immobilized receptor ectodomains. The biotinylated proteins were fixed separately to a streptavidin-coated matrix of a biosensor CM5 in flow cells 2, 3, and 4 at a density of approximately 200 resonance unit (RU), which corresponds to 200 pg protein (approximately 15 fmol of receptor) per mm^2. BMP-2 muteins in concentrations of 15 to 30 nM in HBS buffer (10 mM HEPES, pH 7.4, 500 mM NaCl, 3.4 mM EDTA, 0.005% P20 (Biacore)) were perfused over flow cells 1, 2, 3, and 4 in series at a flow rate of 10 µl/min at 25ºC, and sensograms were recorded at a data sampling rate of 2.5 Hz. The association period was 20 minutes and the dissociation period was set to 6 minutes. Free receptors were regenerated by perfusion with 0.1 M acetic acid, 1 M NaCl for 2 minutes. The base sensogram, which had been recorded for flow cell 1 (streptavidin control), was subtracted from the sensograms that had been obtained for flow cells 2 (BMPR-II), 3 (ActR-II), and 4 (BMPR-IA). The differential sensograms were evaluated in accordance with the fitting route 2 provided by the BIA evaluation software 2.2.4 (Biacore). Equilibrium binding of BMP-2 muteins at a concentration of 45 nM (EQ_{45}) was measured twice in duplicate with a maximum standard deviation (SD) of +/- 20%. The reported rate constants k_{on} for association rate and k_{off} for dissociation for the interaction between BMPR-IA and BMP-2 muteins are mean values obtained from at least 12 measurements, which were conducted with at least three different concentrations of ligands. The standard deviations were 13% for k_{on} and 19% for k_{off}. As the true stoichiometry of complex formation is still uncertain, all sensograms were evaluated on the basis of an unconfirmed 1:1 association model and therefore only apparent, but no absolute constants are listed.

Example 1

Selection of BMP-2 muteins

[0083] In order to identify functionally important amino acid side chains and receptor-binding epitopes in the mature part of human BMP-2, 57 amino acid residues were individually substituted by in vitro mutagenesis (Kirsch et al., 2000 (b)). The substituted residues are shown in FIG. 1 over the BMP-2 sequence. The muteins were expressed in E. coli. A set of 42 muteins was obtained, which were substituted at 40 different positions. Expression in E. coli resulted in dimeric proteins, which were obtained with a purity of greater than 95% and in yields sufficient for the subsequent analysis of biological activity and receptor binding.

[0084] In a first round of mutagenesis, 20 residues having side chains exposed at the surface of the molecule were selected, which span the entire surface of the BMP-2 in a mesh-like manner. After obtaining muteins with promising phenotypes, juxtaposed surface residues were systematically replaced. Initially, residues were substituted with alanine in order to be able to estimate the contribution of the replaced side chain to binding energy. Later, the amino acid residues were replaced with charged residues in order to observe the change of the phenotypical properties after introduction of a charge.

Example 2

Biological Activity of BMP-2 Muteins

[0085] The C2C12 cell assay, which had been used for the quantitative determination of the biological activity of BMP-2 muteins, allows relatively small alterations to be established reproducibly. Mice promyoblast cells differentiate very quickly into multinuclear myotubes under hunger conditions. BMP-2 inhibits this myogenic path and induces the formation of osteoblast-like cells, which are positive for alkaline phosphatase (ALP). Depending on the dose, BMP-2 induces high alkaline phosphatase (ALP) activity in starved C2C12 cells with an ED_{50} of 20 +/- 10 nM (FIG. 2B). The functional significance of BMPR-IA for the osteoinductive effects of BMP-2 in C2C12 cells is already known. The BMPR-IB receptor is detected only at very low levels and is therefore unlikely to play a functional role in these cells. The type II receptors BMPR-II and ActR-II are present in C2C12 cells and can be cross-linked to the BMP-2 ligand in the absence, and more efficiently in the presence, of BMPR-IA. To date it has not been established with certainty if both type II receptors mediate the BMP-2 responses in host cells. Several BMP-2 muteins exhibited clearly reduced activity when analyzed at a concentration of 250 nM for C1C12 cells (FIG. 2A). Muteins A34D and L90A induce no significant response at all. Several other mutant proteins exhibited reduced activity ranging from 2% to 30% of the BMP-2 activity. The symbols indicating the activity of the individual proteins at a concentration of 250 nM are colored according the results of a receptor interaction analysis (see below). Red symbols indicate reduced affinity for the BMPR-II ectodomain, while blue symbols denote an altered interaction with the BMPR-IA ectodomain.

[0086] Representative examples of muteins having approximately 50% (D30K), less than 10% (P50A), and less than 1% (A34D) residual activity are shown in FIG. 2c by efficiency curves. Dosages above 250 nM were not analyzed because the BMP-2 proteins precipitate in the culture medium at concentrations above 500 nM.

[0087] The changes in the biological activity of the muteins described may be caused by significant changes in the structure, stability, or solubility of the protein as a result of amino acid substitutions. Alternatively, functional side chains involved in binding of the type I or type II BMP-2 receptor may have become impaired. The latter possibility, which is to say that specific alterations were produced in the muteins, will be examined in the experiments described below.

Example 3

Antagonist Activity

[0088] Surprisingly, several of the muteins were able to inhibit BMP-2 activity at concentrations of 10 to 250 nM. When C2C12 cells were stimulated with a constant quantity of BMP-2 in the presence of 250 nM mutein, the induction of ALP activity was found to be reduced to less than 1% by the mutein A34D, to approximately 3% by L90A, to approximately 20% by L100A, and to 80% by S88A of the value induced by BMP-2 in the absence of mutant proteins (FIG. 2c).

[0089] The inhibitory properties of these antagonists/partial agonists were confirmed by determining the dose/inhibitions lines, as illustrated in FIG. 2D. The mutant proteins A34D,

L90A, and L100A inhibited half-maximally at concentrations of 20 to 40 nM. This IC_{50} value is similar to a concentration of 10 nM BMP-2 during the assay. Accordingly, the inhibitory muteins operate at concentrations similar to BMP-2, wherein they most likely compete with BMP-2 for a common receptor-binding site.

[0090] The detection of BMP-2 muteins having antagonistic and/or partially agonistic properties indicates that the respective amino acid substitutions produce specific alterations that impair the potency of the BMP-2 protein, but leave receptor binding affinity largely unaffected.

Example 4

Interaction of BMP-2 Muteins with Receptor Ectodomains

[0091] It is already known that type I BMP-2 receptors BMPR-IA and type II receptors BMPR-II and ActR-II are present in C2C12 cells, which seem to mediate the BMP-2 responses. As a result, there was the remote possibility that the functional alterations observed in several of the BMP-2 muteins could result from specific alterations in the BMP-2 epitopes for binding of these receptor chains. This hypothesis was explored in detail by interaction analysis, in which recombinant ectodomains of BMPR-IA, BMPR-II, and ActR-II were employed. Quantitative radioligand binding experiments for exploring binding of BMP-2 muteins to entire cells would have been difficult, since the BMP-2 protein binds to the glycosaminglycans present in the extracellular matrix and at the cell surfaces. The interaction between the recombinant receptor and ectodomains was recorded using a biosensor system. It was already shown for other receptor systems that minor changes in the binding affinity or kinetics of ligand binding can be demonstrated employing receptor domains immobilized on a biosensor system. It has been shown that the BMPR-II receptor proteins immobilized on the biosensor are very stable and survive several dozen cycles of ligand binding and dissociation, without changing the binding properties. For this reason, kinetics and equilibrium binding of all BMP-2 proteins were measured under equal conditions. In this way, it was possible to readily establish differences between the BMP-2 and muteins, even if the kinetics values and equilibrium constant values are more relative values.

[0092] Sensograms were recorded and evaluated, as those shown in FIG. 3. Employing immobilized BMPR-IA ectodomains, it was possible to readily analyze differences in the rate constants of complex formation (k_{on}) and dissociation (k_{off}) with BMP-2 muteins, which is shown in FIG. 3A for sensograms recorded for a 45 nM concentration of the muteins. The concentration dependence of BMP-2 equilibrium binding, as shown in FIG. 4a, yields an apparent K_d of approximately 1 nM. This affinity is in the range of high-affinity binding, which was observed, for example, between hGH and hGHbp or IL-4 and IL-4Rα, and is primarily due to the slow dissociation rate of the ligands (apparent $k_{off} \cong 4 \times 10^{-4}\ s^{-1}$), which means a half-life value of the complex of approximately 0.5 hour. It was not possible to conclusively establish whether this extraordinarily long half-life value was caused by the simultaneous interaction of BMP-2 with two immobilized receptors, or resulted from a true 1:1 interaction. The associate rate (apparent $k_{on} \cong 7 \times 10^{-5}\ M^1\ s^1$) is comparable with that of other receptors. A set of muteins exhibited a specifically increased dissociation rate of the complex with BMPR-IA, wherein the k_{off} values were 2 to 5 times greater than those of BMP-2 (see FIG. 4C, light blue symbols). Two muteins modified at position D30 (D30A, D30K) and two

muteins at position W31 (W31A, W31C) are part of this subset. Another subset of four muteins exhibited decreased rate constants for the association with the BMPR-IA ectodomain, with K_{on} values that were 5 to 10 times lower than those of BMP-2 (FIG. 4C, dark blue symbols). The reduced k_{on} values of V26A, F49A, P50A und H54D were observed only for BMPR-IA, but not for BMPR-II or ActR-II interactions and therefore cannot be due to instability or impurity, which is to say lower effective concentrations of these muteins. Two muteins, specifically K101E and Y103A, exhibited a 2-fold alteration in both k_{on} and k_{off}. The kinetics of the interaction with BMPR-IA of the other BMP-2 muteins did not differ significantly from the kinetics of BMP-2.

[0093] Binding of BMP-2 and BMP-2 muteins to the BMPR-II ectodomain was also recorded, despite the low affinity of this interaction (FIG. 3B). An apparent dissociation rate K_d of approximately 100 nM was derived from the concentration dependence of the equilibrium binding as shown in FIG. 4A. The affinity of BMP-2 for the ActR-II ectodomain was found to be marginally higher ($K_d \cong 50$ nM). In contrast, the apparent K_d for activin binding to the ActR-II ectodomain was reported to be 2-7 nM (Donaldson et al., 1999). The sensograms shown in FIG. 3B demonstrate that the muteins exhibit clear differences with respect to equilibrium binding to the type II receptor BMPR-II. Binding of the mutein A34D was 5 times weaker than that of BMP-2 or the muteins D30K and P40A. The kinetic constants for the interaction between BMP-2 and the type II receptors are relatively high ($k_{on} > 10^6$ M^1 s^1; $k_{off} > 10^2$ s^1). This prevented a reliable evaluation of the k_{on} or k_{off} values. A specific subset of muteins, which included substitutions at five different positions, exhibited equilibrium binding (EQ_{45}) to the BMPR-II ectodomain which was 3 to 15 times lower than that of BMP-2 (FIG. 4D; solid symbols). These deviations were specific to BMPR-II interaction. The k_{on} values of these muteins for BMPR-IA binding were normal.

Example 5

Binding Determinants and Antagonist/Agonist Activity of BMP-2 Muteins

[0094] It was observed that muteins having low affinity for BMPR-II behave as competitive inhibitors of BMP-2 during the C2C12 assay. The determinants of the BMP-2 muteins for ActR-II binding differ from those of BMPR-II (FIG. 4B). The mutein H39D exhibited no reduced, and the muteins A34D and L90A exhibited only a 2-fold decrease in, ActR-II affinity. The binding affinity of S88A and L100A was altered similarly for both type II receptor chains. Such differential effects of amino acid substitutions for binding to different receptors allows the construction of selective agonists activating one or the other receptor chain.

[0095] Unlike in other receptor systems, no binding hot spots were observed in the present system. At most a 5- to 30-fold alteration in kinetic or equilibrium constants was observed in several muteins. However, there is the possibility that several of the main determinants are not represented in the present collection of BMP-2 muteins. These possibly missing determinants may be among the residues which were not possible to express or obtain after substitution yielding proteins. Examples could be G27 and W28. Another more likely possibility, however, is that hydrogen bridges involving the N or O atoms of the main peptide bonds interact with the receptors, thereby contributing to binding affinity.

[0096] In addition, it is interesting that the biological affinity of several muteins was considerably reduced, despite the minor alterations in binding affinity. It cannot be excluded

that several muteins are destabilized by an amino acid substitution and are partially inactivated during the 3-day incubation as part of the C2C12 assay. This, however, cannot apply to the antagonistic muteins, which in the case of A34D, for example, have a 100-fold reduced biological activity. In contrast, it is more likely that the discrepancy in the extent of alterations during physical receptor binding and cellular activity in several of the muteins is caused by avidity effects during the interaction of the multiple binding epitopes of BMP-2 with multimeric cellular receptors in the membrane.

Example 6

Localization of Binding Epitopes

[0097] The amino acid positions of BMP-2 determining the binding affinity for either the BMPR-IA or the BMPR-II receptor chains are part of two non-overlapping subsets. As depicted in FIG. 1, these determinants are distributed along the entire BMP-2 sequence. The space-filling model (Scheufler et al., 1999) in FIG. 5, however, indicates that the functional residues form two separate epitopes on the surface of the homodimeric BMP-2 molecule.

[0098] The determinants for the BMPR-IA interaction co-localize in the wrist epitope, which comprises residues of subunit 1 (italicized letters) and of subunit 2 (normal letters). One monomer provides the residues V26, D30, and W31 in the long loop connecting the sheets β-2 and β-3 and the weak determinants K101 and Y103 localized in sheet β-8. The other monomer contributes residues I62, L66, L66, and N68 in helix α3 and residues F49, P50, and H54 in the long loop in front of helix α3. Remarkably, the muteins having substitutions in the latter loop have reduced association rate constants. This may be related to the observation that residues in this loop had the highest B-factors of 40-90. The B-factors are a measure of the order of atoms in the crystal and of the accuracy with which the atoms are defined in the protein model. As a result, the B-factors are indirectly a measure of the mobility of the atoms in the protein crystal. Even higher mobility of this loop in the muteins could decrease the likelihood of a productive encounter with BMPR-IA, thereby decelerating association. The weak determinant H17 seems to be separated from the other functional wrist epitope residues. It is possible that BMP-2 amino acid residues that have not yet been analyzed and are located between H17 and H54 may constitute further contact sites for BMPR-IA.

[0099] The knuckle epitope of BMP-2 involved in binding of BMPR-II comprises the residues of only one subunit. The residues A34 and H38 occur in the sheet β3 or β4, while S88 and L90 occur in β7 and L100 in β8. Residue E109, which was found to yield a mutein having higher affinity for BMPR-II after arginine substitution, may be a further contact residue. The knuckle epitope seems to be smaller than the wrist epitope, since many residues at the boundary to the knuckle epitope were able to be altered without any detectable effect on receptor binding by [sic] biological activity. It cannot be excluded that the knuckle epitope comprises further functional residues.

[0100] The homodimeric BMP-2 protein has a two-fold symmetry axis, which in FIG. 5 is located in the plane of the paper and extends from top to bottom. On the back of the protein, thus a second pair of wrist and knuckle epitopes is present.

Example 7

Combination of Amino Acid Substitutions in BMP-2 Double Mutants

[0101] The simultaneous substitution of two amino acids involved in the low-affinity binding to BMPR-II considerably amplifies the effects on biological activity. As is apparent from Table III below, the inhibitory activity of bi-substituted muteins was significantly elevated. Surprisingly, it was found that the combination protein A34D/D53A, in which both an amino acid in the wrist epitope and an amino acid in the knuckle epitope were substituted, in the presence of 20 nM BMP-2 has the highest antagonistic activity of all muteins analyzed. This could be due to the fact that the D53A substitution (wrist epitope) in the mono-substituted mutein causes an increase in BMPR-IA affinity (and also in biological activity), however at the same time the interaction with BMPR-II required for biological activity is efficiently weakened by A34D.
[0102] Overall, however, it has been shown that the combination of two mutations in the region of the amino acids in charge of low-affinity binding produces a significant antagonistic effect.

Table III

[0103] Elevated effects for the kinetic constants k_{on} and k_{off} for complex association and dissociation with the BMPR-IA ectodomain; equilibrium binding at 45 nM concentrations, EQ_{45}, to BMPR-II and ActR-II ectodomains. The activity of alkaline phosphatase in the presence of 250 nM mutein, ALP (250), was determined in the absence and presence of 10 nM or 20 nM BMP-2.

Mutein	Epitope	BMPR-IA		BMPR-II	ActR-II		ALP (25)		
		k_{on}	k_{off}	EQ_{45}			-	+10 nM BMP-2	+20 nM BMP-2
		(var)/(wt)					(% BMP-2)		
BMP-2		1.0	1.0	0.99	1.0		100	320	220
H39D	2	1.1	0.79	0.24	0.79		18	100	82
S88A	2	1.1	0.78	0.29	0.32		2.4	65	71
L100A	2	1.2	0.81	0.22	0.34		2.0	32	18
H39D/S88A	2/2	1.2	0.88	0.09	0.35		<0.5	34	50
H39D/L100A	2/2	1.1	0.90	0.02	0.33		0.6	2.6	4.2
D30A	1	3.0	0.97	1.0	1.0		62	330	220
A34D	2	0.56	0.56	0.06	0.38		<0.5	<0.5	2.9
D53A	1	1.1	1.2	0.99	1.2		130		
D30A/A34D	1/2	1.9	0.85	0.02	0.31		<0.5	2.2	7.4
A34D/D53A	2/1	1.1	1.5	<0.02	0.31		<0.5	<0.5	0.7

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SEQUENCE PROTOCOL

<110> Sebald, Walter

<120> Mutein of a chain of a protein from the superfamily of the growth factor TGF-beta

(See original for sequence listings)

Claims

1. A mutein of a chain of a protein from the superfamily of the growth factor TGF-β, wherein the mutein exhibits an antagonistic and/or partially agonistic activity after forming a homodimer, and wherein the mutein is altered at one or more positions thereof, which in the unaltered protein are involved in a low-affinity binding to the receptor thereof.
2. The mutein according to claim 1, wherein the mutein is altered by deletion and/or substitution and/or insertion and/or modification of one or more amino acids.
3. The mutein according to claim 1 or 2, wherein the mutein is derived from a chain of a BMP-2-like protein and, in the mutein, one or more positions in the knuckle epitope are altered.
4. The mutein according to claim 3, wherein the BMP-2-like protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, GDF-5, GDF-6, and GDF-7.
5. A mutein according to any one of claims 3 or 4, characterized in that one or more amino acids, selected from the group of amino acids forming the surface-exposed regions in the β-sheet structures β3, β4, β7, β8 and/or β9, are deleted and/or substituted and/or modified and/or that at least one of said surface-exposed regions is altered by the insertion of one or more amino acids.
6. A mutein according to any one of claims 3 or 4, characterized in that at least one of the amino acids connecting the β-sheets β3 and β4 is deleted and/or substituted and/or modified

and/or that one or more amino acids are inserted into the amino acid region connecting the β-sheets β3 and β4.

7. A mutein according to any one of claims 3 to 6, characterized in that at least one of the following amino acids is deleted, substituted and/or modified, wherein the position information relates to BMP-2:
V33 A34 P35 P36 G37 Y38 H39 F41 Y42 T82 E83 L84 S85 A86 I87 S88 L90 K97 V98 V99 L100 V107 E109 and G110.

8. A mutein according to any one of claims 1 to 7, characterized in that it has at least 50% identity with a chain of a BMP-2-like growth factor from the TGF-β superfamily and exhibits antagonistic and/or partially agonistic activity.

9. A mutein according to any one of claims 1 or 2, wherein the mutein is derived from a protein of the TGF-β/activin family and, in the mutein, one or more positions in the wrist epitope are altered.

10. The mutein according to claim 9, wherein the protein of the TGF-β/activin family is selected from the group consisting of TGF-β1, TGF-β2, TGF-β3, activins, inhibins, BMP-11, and GDF-8.

11. A mutein according to any one of claims 9 or 10, characterized in that at least one amino acid, selected from the group of amino acids forming the surface-exposed regions in the helix in front of the β-sheet structure β1, the sheet structure β1, the long loop between the sheet structures β2 and β3, the loop in front of helix α3, helix α3, and the sheet structure β8, are deleted and/or substituted and/or modified and/or that at least one of said surface-exposed regions is altered by the insertion of one or more amino acids

12. A mutein according to any one of claims 9 to 11, characterized in that at least one of the following amino acids is deleted, substituted and/or modified, wherein the position information relates to BMP-2:
K5, S13, V26, G27, W28, N29, D30, W31, P48, F49, P50, A52, D53, H54, N59, I62, V63, L66, N68, S69, V70, K101, and Y103.

13. A mutein according to any one of claims 1 to 12, characterized in that it has at least 50% identity with a chain of a TGF-β/activin-like growth factor from the TGF-β superfamily and exhibits antagonistic and/or partially agonistic activity.

14. A mutein according to any one of claims 1 to 13, characterized in that it is covalently bonded to a target-specific molecule.

15. The mutein according to claim 14, characterized in that the target-specific molecule forms a fusion protein with the mutein.

16. A derivative of a protein from the TGF-β superfamily, comprising a mutein according to any one of claims 1 to 15 and a further chain of a protein from the group of the TGF-β superfamily, or a further mutein according to any one of claims 1 to 15.

17. The derivative according to claim 16, comprising a homodimer comprising muteins according to any one of claims 1 to 15.

18. The derivative according to claim 16, comprising a heterodimer comprising muteins according to any one of claims 1 to 15.

19. A pharmaceutical composition, comprising at least one mutein according to any one of claims 1 to 15 and/or a derivative according to any one of claims 16 to 18 and/or a pharmaceutically acceptable salt thereof.

20. Use of a mutein according to any one of claims 1 to 15 and/or a derivative according to any one of claims 16 to 18 for producing a composition for the prophylaxis and/or treatment of diseases induced by a protein from the superfamily of the growth factor TGF-β .
21. An antibody against a mutein according to any one of claims 1 to 15 or a derivative according to any one of claims 16 to 18.
22. A nucleic acid, comprising a nucleic acid sequence, selected from:
(i) a nucleic acid sequence coding for a mutein according to any one of claims 1 to 15;
(ii) a nucleic acid sequence complementary to the nucleic acid sequence according to (i), and
(iii) a nucleic acid sequence hybridized with a nucleic acid sequence according to (ii) and coding for a mutein exhibiting antagonistic or partially agonistic activity after forming a homodimer.
23. The nucleic acid according to claim 22, characterized in that the hybridization according to (iii) is carried out under stringent conditions.
24. The nucleic acid according to claim 19, characterized in that the nucleic acid is genomic DNA, cDNA, synthetic DNA, or RNA.
25. A nucleic acid according to any one of the claims 22 to 24, further comprising a promoter suited for expression control, wherein the nucleic acid sequence coding for a mutein is controlled by the promoter.
26. A vector, comprising a nucleic acid according to any one of claims 22 to 28.
27. A host organism, comprising a nucleic acid according to any one of the claims 22 to 25 or a vector according to claim 26.
28. A method for producing a mutein according to any one of claims 1-15, comprising the cultivation of a host organism according to claim 27 under conditions suitable for expression and optionally the purification of the expressed mutein.

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Drawings Page [#]

Number: **DE 100 26 713 A1**
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Disclosure date: December 6, 2001

(See original for actual figures)

FIG. 2A:
Sequenz-Position Sequence position
Induktion der Aktivität von alkalischer Phosphatase [%BMP-2] Induction of activity of alkaline phosphatase [%BMP-2]

FIG. 2B:
BMP-2 Mutein [µM] BMP-2 mutein [µM]
Induktion der Aktivität von alkalischer Phosphatase [%BMP-2] Induction of activity of alkaline phosphatase [%BMP-2]

FIG. 2C:
Sequenz-Position Sequence position
BMP-induzierte Aktivität von alkalischer Phosphatase (%) BMP-inducted activity of alkaline phosphatas(%)

FIG. 2D:
BMP-2 Mutein [µM] BMP-2 mutein [µM]
BMP-induzierte Aktivität von alkalischer Phosphatase (%) BMP-inducted activity of alkaline phosphatas(%)

FIG. 3A:
Antwort [RU] Reponse [RU]
Zeit [s] Time [s]

FIG. 3B:
Antwort [RU] Reponse [RU]
Zeit [s] Time [s]

FIG. 4A:
Gleichgewichtsbindung [RU] Equilibrium binding [RU]